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CONTRACT NO: DAMD17-91-C-1106

TITLE: UNIQUE ENZYME TARGETS FOR GONOCOCCAL ANTIMETABOLITES

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13. ABSTRACT (Maximum 200 words)

A rationale exists in support of the possibility that the lactate dehydrogenase isoenzymes of *Neisseria gonorrhoeae* might be essential enzymes for maintaining the host-pathogen relationship. Specific aims for Phase I were to employ lactate dehydrogenase mutants and selective inhibitors of the membrane-bound lactate dehydrogenase (iLDH) and soluble lactate dehydrogenase (nLDH) in genetic and physiological experiments to gain an understanding of the redox relationships and overall biochemical roles of iLDH and nLDH. Success in this approach would provide the basis for a Phase II project for determining the gene sequences and for the design of precise inhibitors acting at the enzyme, or perhaps the gene level. These inhibitors can then be deployed in model systems of pathogenesis.

14. SUBJECT TERMS
Gonococci; Lactate Dehydrogenase; Pathogenicity; Enzyme Inhibitors; Antimetabolites; SBIR; RAD I

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A. Lactate Dehydrogenase Mutants

Mutant strains lacking the broad-specificity (utilizes phenyllactate, 4-hydroxyphenyllactate and prephenyllactate in addition to D(-) and L(+)-lactate) iLDH (designated in this work as iLDH-I) were further mutagenized with EMS with the goal of obtaining mutants that would be unable to utilize lactate as carbon source. The iLDH-I mutants were selected as phenyllactate-resistant derivatives of a strain of *N. gonorrhoeae* that is growth-inhibited by exogenous phenylalanine. Although these mutants manifest a competitive disadvantage in a neutrophil system, they are still able to utilize lactate. We assumed at the initiation of this project that the nLDH, normally expected to function as pyruvate reductase, can (in the absence of iLDH) function in the catabolic direction. However, we have found that all the mutants resulting from EMS mutagenesis which were unable to grow on lactate retained activity for nLDH. Characterization of growth of these mutants on glucose, pyruvate and acetate as carbon sources revealed no differences compared to the iLDH-I mutant, except for morphological pleiotropism and a slower growth rate which was most evident with glucose. Evaluation of lactate dehydrogenase activities of the parental strain of the iLDH-I mutant, the iLDH-I mutant and the lactate (-) mutants derived from the iLDH-I mutant following growth on glucose or lactate (when applicable) raised the possibility that a second iLDH, in addition to iLDH-I, may have gone unrecognized because of repression by growth on glucose. Specific activity for iLDH (pyridine-independent) in the iLDH-I mutant increased several-fold when lactate was supplied as carbon source as compared to a negligible level found in glucose-grown cells. Similarly, the specific activity for total iLDH increased 40-50% when the parental strain of the iLDH-I mutant was grown with lactate rather than glucose as carbon source. No iLDH activity, not even the negligible level characteristic of the iLDH-I mutant grown on glucose, was detected in the lactate (-) mutants derived from the mutant lacking iLDH-I. Interestingly, the specificity of the iLDH activity found in the lactate-grown iLDH-I mutant was quite different from iLDH-I characterized from wildtype. This enzyme preferred D(-)-lactate, used L(+)-lactate only poorly (<1/20 as well as D(-)-lactate), and did not use either phenyllactate or 4-hydroxyphenyllactate as substrate. In contrast, iLDH-I prefers L(+)-lactate, utilising D(-)-lactate only about 30% as well. Furthermore, iLDH-I uses phenyllactate and 4-hydroxyphenyllactate at approximately the same efficiency as D(-)-lactate. Ion-exchange chromatography of protein extracts from wildtype grown with lactate as carbon source confirmed the presence of a second peak of iLDH activity, well separated from iLDH-I. The second peak of iLDH activity (designated as iLDH-II) showed similar properties to the iLDH activity characterized in the iLDH-I mutant grown on lactate. These data indicate that the nLDH plays no apparent role in the catabolism of lactate. Rather, two isoenzymes of iLDH, each of which exhibits unique substrate specificities and physical characteristics, appear to be responsible for repeated failures to isolate lactate-deficient

3. DEPARTMENT OF DEFENSE

SMALL BUSINESS INNOVATION RESEARCH (SBIR) PROGRAM
PROJECT SUMMARY

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TOPIC NUMBER: A91-027

PROPOSAL TITLE: Unique Enzyme Targets For Gonococcal Antimetabolites

Contract No. DAMD17-91-C-1106

FIRM NAME: MetaGene Corporation

PHASE I or II PROPOSAL: Phase I FINAL REPORT

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

The ability of *Neisseria gonorrhoeae* to metabolize lactate derived from host neutrophils has been implicated in a pathogenic mechanism that favors the gonococci and reduces the capabilities of the phagocytes. Approaches employing genetics, physiology, enzymology and chemistry have been utilized to elucidate gene-enzyme relationships of lactate metabolism to gain insight into the nature of the host-pathogen interaction in order to ultimately disrupt the pathogenic state. Two membrane-bound lactate dehydrogenase (iLDH) enzymes [convert lactate to pyruvate] and a soluble lactate dehydrogenase (nLDH) [converts pyruvate to lactate] have been found to participate in lactate metabolism. Both iLDH enzymes and the nLDH appear to be unidirectional. In contrast to the nLDH, the iLDH enzymes are particulate and pyridine-nucleotide independent. Following separation by ion-exchange chromatography the iLDH enzymes were further distinguished on the basis of preference for D(-) or L(+)-lactate. The enzyme, designated iLDH-I, has an unusual breadth of substrate range enabling it to oxidize a variety of lactyl-derivatives. Only mutants lacking both iLDH activities were unable to utilize lactate as carbon source. Inhibitors targeted to iLDH-I have been synthesized which inhibit the growth of wildtype but not mutants devoid of iLDH-I.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

Potential application of this research may provide alternative strategies to current treatments of this pathogen. Development of new antimicrobials active against gonococci will be beneficial in view of the perpetuity of this pathogen worldwide and the increasing incidence of strains having acquired resistance to contemporary antibiotics.

List a maximum of 8 Key Words that describe the Project.

Gonococci	Antimetabolites
Lactate Dehydrogenase	
Pathogenicity	
Enzyme Inhibitors	

I. PROJECT OBJECTIVES

A rationale exists in support of the possibility that the lactate dehydrogenase isoenzymes of *Neisseria gonorrhoeae* might be essential enzymes for maintaining the host-pathogen relationship. Specific aims for Phase I were to employ lactate dehydrogenase mutants and selective inhibitors of the membrane-bound lactate dehydrogenase (iLDH) and soluble lactate dehydrogenase (nLDH) in genetic and physiological experiments to gain an understanding of the redox relationships and overall biochemical roles of iLDH and nLDH. Success in this approach would provide the basis for a Phase II project for determining the gene sequences and for the design of precise inhibitors acting at the enzyme, or perhaps the gene level. These inhibitors can then be deployed in model systems of pathogenesis.

A. Specific Objectives

(1) Construction of lactate dehydrogenase mutants of *N. gonorrhoeae* unable to utilize lactate. A double iLDH/nLDH and a single nLDH mutant were to be constructed from an iLDH mutant. Growth of the mutants would be compared under differing conditions of carbon-source nutrition.

(2) Synthesis of inhibitors of iLDH. Derivatives of phenyllactate, 4-hydroxyphenyllactate and/or prephenyllactate would be prepared with the aim of optimizing stability and transport into cells, of obtaining the desired specificity characterisic with respect to the iLDH target, and to introduce reactive moieties that may produce irreversible inhibitors. Derivatives would be evaluated by *in vitro* enzyme assay with regards to the ability to act as substrate or inhibitor of the conversion of lactate to pyruvate.

(3) Evaluation of inhibitors of iLDH activity for ability to prevent growth of lactate dehydrogenase mutants retaining iLDH function.

(4) Evaluation of inhibitors showing antimicrobial action against lactate dehydrogenase mutants retaining iLDH function for ability to inhibit growth of wildtype (parental) strain.

II. WORK COMPLETED/RESULTS OBTAINED

We have achieved all the major objectives enumerated at the beginning of this project. The increasing complexity of lactate metabolism in *N. gonorrhoeae* revealed during this project, however, required that we redirect some of the minor project objectives. These objectives have also been realized.

A. Lactate Dehydrogenase Mutants

Mutant strains lacking the broad-specificity (utilizes phenyllactate, 4-hydroxyphenyllactate and prephenyllactate in addition to D(-) and L(+)-lactate) iLDH (designated in this work as iLDH-I) were further mutagenized with EMS with the goal of obtaining mutants that would be unable to utilize lactate as carbon source. The iLDH-I mutants were selected as phenyllactate-resistant derivatives of a strain of *N. gonorrhoeae* that is growth-inhibited by exogenous phenylalanine. Although these mutants manifest a competitive disadvantage in a neutrophil system, they are still able to utilize lactate. We assumed at the initiation of this project that the nLDH, normally expected to function as pyruvate reductase, can (in the absence of iLDH) function in the catabolic direction. However, we have found that all the mutants resulting from EMS mutagenesis which were unable to grow on lactate retained activity for nLDH. Characterization of growth of these mutants on glucose, pyruvate and acetate as carbon sources revealed no differences compared to the iLDH-I mutant, except for morphological pleiotropism and a slower growth rate which was most evident with glucose. Evaluation of lactate dehydrogenase activities of the parental strain of the iLDH-I mutant, the iLDH-I mutant and the lactate (-) mutants derived from the iLDH-I mutant following growth on glucose or lactate (when applicable) raised the possibility that a second iLDH, in addition to iLDH-I, may have gone unrecognized because of repression by growth on glucose. Specific activity for iLDH (pyridine-independent) in the iLDH-I mutant increased several-fold when lactate was supplied as carbon source as compared to a negligible level found in glucose-grown cells. Similarly, the specific activity for total iLDH increased 40-50% when the parental strain of the iLDH-I mutant was grown with lactate rather than glucose as carbon source. No iLDH activity, not even the negligible level characteristic of the iLDH-I mutant grown on glucose, was detected in the lactate (-) mutants derived from the mutant lacking iLDH-I. Interestingly, the specificity of the iLDH activity found in the lactate-grown iLDH-I mutant was quite different from iLDH-I characterized from wildtype. This enzyme preferred D(-)-lactate, used L(+)-lactate only poorly (<1/20 as well as D(-)-lactate), and did not use either phenyllactate or 4-hydroxyphenyllactate as substrate. In contrast, iLDH-I prefers L(+)-lactate, utilizing D(-)-lactate only about 30% as well. Furthermore, iLDH-I uses phenyllactate and 4-hydroxyphenyllactate at approximately the same efficiency as D(-)-lactate. Ion-exchange chromatography of protein extracts from wildtype grown with lactate as carbon source confirmed the presence of a second peak of iLDH activity, well separated from iLDH-I. The second peak of iLDH activity (designated as iLDH-II) showed similar properties to the iLDH activity characterized in the iLDH-I mutant grown on lactate. These data indicate that the nLDH plays no apparent role in the catabolism of lactate. Rather, two isoenzymes of iLDH, each of which exhibits unique substrate specificities and physical characteristics, appear to be responsible for repeated failures to isolate lactate-deficient

mutants directly.

B. Inhibitors of iLDH

The iLDH-I of *N. gonorrhoeae* is unusual in its broad substrate specificity whereby phenyllactate and 4-hydroxyphenyllactate can be utilized in place of lactate. The only other example of a lactate dehydrogenase which utilizes 4-hydroxyphenyllactate is found in *Candida maltosa*. This lactate dehydrogenase utilizes 4-hydroxyphenyllactate 10 times as well as lactate. Since 4-hydroxyphenyllactate is a normal host metabolite, the breadth of substrate recognition by iLDH-I may be important *in vivo*. In order to exploit this possibility we have carried out a survey with a series of analog structures of phenyllactate and 4-hydroxyphenyllactate in order to determine molecular features that affect binding. Three classes of analogs including *para*-substituted phenyllactate, *ortho*-substituted phenyllactate, and a C-3 modification of the lactyl moiety were synthesized and evaluated for ability to inhibit iLDH-I function and to be used as substrate. The four *para*-substituted derivatives of phenyllactate (nitro > methoxy > bromo = chloro) inhibited iLDH-I activity by 20-50% when inhibitor stoichiometries with respect to substrate (DL-lactate) were 1:10. The three *ortho*-substituted derivatives of phenyllactate (bromo = chloro > methoxy) inhibited iLDH-I activity by 40-50% when inhibitor stoichiometries with respect to substrate were 1:4-8. The C-3 modification of the lactyl moiety of phenyllactate rather than the aromatic ring yielded the inhibitor, 3-phenyl-2-hydroxypentanoic acid, which inhibited iLDH-I activity by greater than 50% when the inhibitor to substrate stoichiometries were 1 to 5, respectively. Despite the success of synthesizing analogs which were able to efficiently inhibit function of iLDH-I, none of the various inhibitors showed detectable ability to be used as substrate by iLDH-I *in vitro* or to inhibit iLDH-II activity.

C. Utilization of Inhibitors of iLDH-I as Antimetabolites

All the inhibitors of iLDH-I function listed above were tested for ability to inhibit growth of wildtype and the iLDH-I mutant strains when grown on either glucose or lactate as carbon source. Two analogs, 4-chlorophenyllactate and 2-chlorophenyllactate, were able to produce significant inhibition of growth of wildtype grown on either glucose or lactate as carbon source. The *ortho*-chloro compound was several-fold more effective than the *para*-chloro derivative, as measured by the zones of inhibition on a agar-disc assay. No inhibition of the iLDH-I mutant (lacks iLDH-I but retains iLDH-II activity) was noted with either of the analogs regardless of the carbon source. None of the other compounds showed any ability to inhibit either of the two strains.

III. TECHNICAL FEASIBILITY

A fundamental approach to the selective attack of the pathogen in a host-pathogen relationship is to identify unique biochemical targets in the pathogen. Metabolic sequences thought to be associated with maintenance of the pathogenic state, such as those carried out by the iLDH-I/iLDH-II system in *N. gonorrhoeae* are potential targets for selective attack. The substrate diversity exhibited by iLDH-I, in contrast to the generally high selectivity of lactate dehydrogenases in nature, makes iLDH-I a promising target for antimetabolites functioning as substrate mimics since toxicity with respect to the host lactate dehydrogenases would not be expected. We have accomplished our goals of : a) constructing a mutant (the iLDH-I/iLDH-II double mutant) which is unable to utilize lactate as carbon source; b) preparing inhibitors of iLDH-I activity which are stable, and selective; and c) developing inhibitors of iLDH-I which show antimicrobial abilities. The experimental plan which we have put forth demonstrates a feasibility for gonococcal treatment based on enzyme targets involved in lactate metabolism in the pathogen. The research completed in Phase I will provide for Phase II: a) a genetic system (iLDH-I/iLDH-II double mutant) for the selection of of cloned iLDH genes; b) insight into the substituent groups capable of yielding antimicrobial abilities to iLDH-I inhibitors; and c) a greater appreciation of genetic perturbations impacting lactate metabolism in *N. gonorrhoeae*. The iLDH-I gene sequence and deduced amino acid sequence which will be obtained in Phase II will allow the design of more precise inhibitors based on computer modeling of protein and inhibitor structure.

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH, DEVELOPMENT, ACQUISITION, AND LOGISTICS COMMAND (PROVISIONAL)
FORT DETRICK, MARYLAND 21702-5012

REPLY TO ATTENTION OF

ERRATA

SGRD-RMI-S (70-1y)

22 JUL 1994

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Bldg. 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research, Development, Acquisition, and Logistics (USAMRDAL) Command (Provisional), has reexamined the need for the limited distribution statement on technical report for Contract No. DAMD17-91-C-1106. Request the limited distribution statement for AD No. ADB176142, be changed to "Approved for public release; distribution unlimited," and that copies of this report be released to the National Technical Information Service.

2. The point of contact for this request is Ms. Virginia Miller, DSN 343-7328.

151, 20/14

ERRATA
ADB176142

CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management